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Effects of proteinase inhibitors on digestive proteinases and growth of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae)

B. Oppert[a,*], T.D. Morgan[a], K. Hartzer[b], B. Lenarcic[c], K. Galesa[c], J. Brzin[c], V. Turk[c], K. Yoza[d], K. Ohtsubo[d], K.J. Kramer[a]

[a]*USDA ARS Grain Marketing and Production Research Center, 1515 College Ave., Manhattan, KS 66502, USA*
[b]*Department of Entomology, Kansas State University, Manhattan, KS 66506, USA*
[c]*Department of Biochemistry and Molecular Biology, J. Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia*
[d]*National Food Research Institute, 2-1-12 Kannon-Dai, Tsukuba Science City, Ibaraki, 305-8642, Japan*

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Abstract

The physiology of the gut lumen of the red flour beetle, *T. castaneum*, was studied to determine the conditions for optimal protein hydrolysis. Although the pH of gut lumen extracts from *T. castaneum* was 6.5, maximum hydrolysis of casein by gut proteinases occurred at pH 4.2. The synthetic substrate *N*-α-benzoyl-DL-arginine-ρ-nitroanilide was hydrolyzed by *T. castaneum* gut proteinases in both acidic and alkaline buffers, whereas hydrolysis of *N*-succinyl-ala-ala-pro-phe ρ-nitroanilide occurred in alkaline buffer. Inhibitors of *T. castaneum* digestive proteinases were examined to identify potential biopesticides for incorporation in transgenic seed. Cysteine proteinase inhibitors from potato, Job's tears, and sea anemone (equistatin) were effective inhibitors of in vitro casein hydrolysis by *T. castaneum* proteinases. Other inhibitors of *T. castaneum* proteinases included leupeptin, L-trans-epoxysuccinylleucylamido [4-guanidino] butane (E-64), tosyl-L-lysine chloromethyl ketone, and antipain. Casein hydrolysis was inhibited weakly by chymostatin, *N*-tosyl-L-phenylalanine chloromethyl ketone, and soybean trypsin inhibitor (Kunitz). The soybean trypsin inhibitor had no significant effect on growth when it was bioassayed alone, but it was effective when used in combination with potato cysteine proteinase inhibitor. In other bioassays with single inhibitors, larval growth was suppressed by the cysteine proteinase inhibitors from potato, Job's tears, or sea anemone. Levels of inhibition were similar to that observed with E-64, although the moles of proteinaceous inhibitor tested were approximately 1000-fold less. These proteinaceous inhibitors are promising candidates for transgenic seed technology to reduce seed damage by *T. castaneum.*

Keywords: Insect digestion; Proteinase inhibitor; Red flour beetle; *Tribolium castaneum*

Abbreviations: BapNA, *N*-α-benzoyl-DL-arginine-ρ-nitroanilide; E-64: L-trans-epoxysuccinylleucylamido [4-guanidino] butane; EDTA, ethylenediamine tetraacetic acid; JCPI, Job's tears cysteine proteinase inhibitor; TLCK, tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; PCPI, potato cysteine proteinase inhibitor; SAAPFpNA, *N*-succinyl-ala-ala-pro-phe ρ-nitroanilide; STI, soybean trypsin inhibitor (Kunitz).

*Corresponding author. Tel.: +785-776-2780; fax: +785-537-5584.
E-mail address: bso@ksu.edu (B. Oppert).

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1. Introduction

Postharvest losses to insect pests are a serious problem for agriculture (Harein and Meronuck, 1995; Cuperus and Krischik, 1995). For example, surveys in 1999 indicated that 71% of Kansas' wheat in storage required treatment with chemical insecticides for protection against stored grain insects (Kolterman et al., 2000). Flour beetles and other pests cause economic losses by feeding on broken grain, grain dust, and milled grain products. The red flour beetle, *Tribolium castaneum* (Herbst), is a serious pest of stored grains and products. A closely related species, the confused flour beetle (*T. confusum*), is the most abundant and injurious pest of flourmills in the US.

Cysteine proteinases are important enzymes for digestion in many coleopteran pests, while vertebrates generally use proteinases from other classes for digestion (Reeck et al., 1997, 1999; Hilder and Boulter, 1999). For this reason, the incorporation of genes encoding cysteine proteinase inhibitors into transgenic grain has been proposed as a method to prevent seed damage from coleopteran pests while posing little or no side effects on vertebrates. One cysteine proteinase, cathepsin-L, has been identified in larvae of the western corn rootworm, *Diabrotica virgifera* (Koiwa et al., 2000). Digestive proteinases of *D. virgifera* were inhibited by cysteine proteinase inhibitors, such as chicken cystatin (Gillikin et al., 1992). Similarly, digestive proteinases in the southern corn rootworm, *Diabrotica undecimpunctata*, were inhibited by 0.1% L-trans-epoxysuccinylleucylamido [4-guanidino] butane (E-64), as well as pepstatin, an acidic proteinase inhibitor (Edmonds et al., 1996) and a potato cysteine proteinase inhibitor (Fabrick et al., 2002). Several coleopterans were susceptible to jackbean canatoxin due to cathepsin-like digestive enzymes that were able to convert the toxin to an active protein (Carlini et al., 1997).

Studies in some coleopterans, however, have suggested that, in addition to cysteine proteinases, a complex pattern of proteinase activities exists in the midgut. Some insect species belonging to the Curculionidae family digest food using a combination of cysteine and serine proteinase classes, as revealed by research on the rice weevil, *Sitophilus oryzae*, and the alfalfa weevil, *Hypera postica* (Chen et al., 1992; Oppert et al., 2000). In the closely related family Chrysomelidae, the watercress leaf beetle, *Phaedon viridus*, has cysteine, serine, and aspartic acid digestive proteinases (Girard et al., 1998). An aspartic acid proteinase with cathepsin D-like specificity was the primary digestive proteinase in the Colorado potato beetle, *Leptinotarsa decemlineata*, with subsequent digestion by cysteine (cathepsin B- and cathepsin H-like) and serine (chymotrypsin-like) proteinases (Brunelle et al., 1999). However, cysteine proteinase inhibitors in the diet were ineffective in suppressing growth because they were inactivated by midgut extracts from *L. decemlineata* (Michaud et al., 1996). Similarly, combinations of cysteine and serine proteinase inhibitors did not affect the growth of *P. viridus* larvae due to rapid degradation by serine proteinases and aminopeptidases.

Digestion in *T. castaneum* appears to be complex in terms of the number and types of proteinases. Two proteinases with pI values approximately pH 3 were identified in *T. castaneum* larvae 20 years ago (Baker, 1982). A cathepsin D-like aspartic acid proteinase was described more recently (Blanco-Labra et al., 1996). Other data support cysteine proteinases as being the major digestive proteinases in *T. castaneum*. Rice and chestnut cystatins significantly reduced the caseinolytic activity of gut extracts from *T. castaneum* (Liang et al., 1991; Chen et al., 1992; Pernas et al., 1998). The growth and development of *T. castaneum* larvae were retarded by inhibitors of cysteine and/or serine proteinases (Birk and Applebaum, 1960; Liang et al., 1991; Chen et al., 1992; Oppert et al., 1993). However, a combination of cysteine and serine proteinase inhibitors was more effective than individual inhibitors in inhibiting growth and causing mortality (Oppert et al., 1993).

To identify enzyme inhibitor genes for potential utilization in the enhancement of seed protection against storage pests, the effects of proteinaceous inhibitors on *T. castaneum* larvae were evaluated. Although some of the proteinaceous inhibitors were similar to E-64 in suppressing larval growth, they were more efficacious when compared on a molar basis. Genes for these inhibitors may be useful for transgenic seed technology to improve seed protection against storage pests such as *T. castaneum*.

2. Materials and methods

2.1. Inhibitors

Inhibitors with different specificities were tested in vitro and in vivo (Table 1). Equistatin was

Table 1
Relative inhibition *in vitro* of casein hydrolysis by *Tribolium castaneum* larval gut proteinases by selected inhibitors

Inhibitor	Target Proteinase(s)[1]	IC_{50} (μM)[2]
Leupeptin	[1]Serine and cysteine proteases, such as trypsin, papain, plasmin, and cathepsin B	0.05 (0.03–0.10)
E-64	[1]Papain and other cysteine proteinases like cathepsin B and L	0.11 (0.02–0.85)
Equistatin	[3]Papain, cathepsin D	0.24 (0.06–0.95)
TLCK	[1]Irreversible inhibitor of trypsin. Can inhibit other serine and cysteine proteases such as bromelain, ficin, and papain	0.35 (0.20–0.63)
JCPI	[4]Cysteine proteases such as papain	0.43 (0.19–0.97)
PCPI	[5]Papain and other cysteine proteinases, like cathepsins B, H, and L	0.76 (0.17–3.30)
Antipain	[1]Papain and trypsin	0.83 (0.28–2.50)
Chymostatin	[1]α, β, γ, δ-chymotrypsin	3.40 (0.65–18.0)
TPCK	[1]Irreversible inhibitor of chymotrypsin. Can inhibit other serine and cysteine proteases such as bromelain, ficin, and papain	24.0 (14.0–41.0)
STI	[1]Trypsin, factor Xa, plasmin, and plasma kallikrein	58.0 (39.0–86.0)
Pepstatin	[1]Aspartic (acid) proteases such as pepsin, renin, cathepsin D, chymosin, and many microbial acid proteases	[6]ND
EDTA	[1]Metalloproteases	[6]NI
Bestatin	[1]Aminopeptidases	NI
Aprotinin	[1]Serine proteases, such as plasmin, kallikrein, trypsin, and chymotrypsin	NI

[1] As per Boehringer Mannheim Proteinase Inhibitors Technical Guide (http://biochem.roche.com).
[2] Concentration resulting in 50% inhibition of the uninhibited activity. 95% confidence interval in parentheses.
[3] Lenarcic and Turk, 1999.
[4] Yoza et al., 2001.
[5] Brzin et al., 1988.
[6] NI, no inhibition at tested doses; ND, could not be determined from data.

purified from the sea anemone, *Actinia equina*, as described by Ritonja et al. (1997) and Lenarcic et al. (1997). A potato cysteine proteinase inhibitor (PCPI) was prepared according to Brzin et al. (1988) and Krizaj et al. (1993). Job's tears cysteine proteinase inhibitor (JCPI) was expressed in *Escherichia coli* as a His-tagged recombinant protein and purified by chromatography using nickel–nitrilotriacetic acid agarose gel (Yoza et al., 2001). Antipain, aprotinin, bestatin, chymostatin, E-64, ethylenediamine tetraacetic acid (EDTA), soybean Kunitz trypsin inhibitor (STI), tosyl-L-lysine chloromethyl ketone (TLCK), and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) were obtained from Sigma Chemical Co., (St. Louis, MO). Leupeptin and pepstatin were obtained from Roche Molecular Biochemicals (Indianapolis, IN).

2.2. Insect dissection

Larvae were from a laboratory culture reared continuously on 95% wheat flour mixed with 5% brewers yeast. At the time of dissection, larvae weighed an average of 3.11 ± 0.50 mg (average of 5 groups of 10 larvae each, mean ± S.D.). Larvae were chilled on ice, the posterior and anterior ends were removed, and entire guts were excised, pooled in deionized water (10 guts/25 μl for proteinase assays, or 10 guts per 50 μl for pH measurements taken prior to freezing), and frozen at −20 °C. For microplate proteinase assays, samples were thawed, vortexed briefly, and the supernatant was collected following centrifugation at $15\,000 \times g$ for 5 min.

2.3. Microplate proteinase assay

Analysis of proteinase activities in vitro was performed with a microplate assay (Oppert et al., 1997). To analyze the effect of pH on proteinase activity, 0.8 gut equivalent was diluted into 90 μl of universal buffer (Frugoni, 1957), composed of phosphoric, acetic, and boric acids mixed with potassium chloride to obtain buffers with pH values from acidic to basic. Because reducing conditions resulted in increased activity, 5 mM L-cysteine was added to all buffers. A general enzyme substrate, fluorescently labeled casein (BODIPY-TR-X casein, Molecular Probes, Eugene, OR), was diluted as per the manufacturer's recommendation, and 10 μl (0.1 μg) was added to each well. Each sample was incubated in triplicate at 37 °C, and the fluorescence was measured (excitation wavelength = 584 nm, emis-

sion wavelength = 620 nm) and corrected by subtracting readings obtained from incubations of substrate only. Measurements of enzyme and buffer or buffer only produced negligible fluorescence.

For inhibition assays, proteinase inhibitors were added to pH 4.2 buffer with 5 mM L-cysteine and preincubated with the gut extract for 30 min at 37 °C prior to the addition of substrate. Inhibition was measured after 4 h incubation at 37 °C. The percent inhibition was calculated using linear regression of data points.

Synthetic substrates conjugated to ρ-nitroanilide obtained from Sigma included *N*-α-benzoyl-L-arginine ρ-nitroanilide (BApNA) and *N*-succinyl ala-ala-pro-phe ρ-nitroanilide (SAAPFpNA). Substrates were diluted to a final concentration of 1 mg/ml in 50 μl of universal buffer with pH values ranging from acidic to basic (Frugoni 1957). To initiate the reaction, 2 gut equivalents of *T. castaneum* larval gut proteinases diluted in 50 μl of the appropriate buffer were added to each well. Triplicate samples were incubated at 37 °C for 5 min, and absorbance at 405 nm was monitored at 15-s intervals. The change in absorbance per min was calculated by KinetiCalc3 software (BIO-TEK, Winooski, VT) and the data were reported in milliabsorbance units per min per gut equivalent, after subtracting the amount of absorbance contributed by autolysis of the substrate.

2.4. pH measurement

Samples containing 10 guts in 50 μl of deionized water were vortexed briefly. Measurements of pH were made using a Hamilton Biotrode filled with Proteolyte connected to a Corning pH meter 440 (Corning, New York).

2.5. Insect bioassay

The bioassay diet consisted of 85% toasted wheat germ (Southland Products, Inc., Lake Village, AR), 10% wheat flour, and 5% brewers yeast (Sigma). This diet was chosen because larvae grew faster than on the rearing diet. In addition, bioassays with this diet reflected the natural situation of larvae feeding primarily on the protein-rich germ of wheat kernels. E-64 was predissolved in water before addition to the diet because of its relatively limited solubility. Proteinaceous inhibitors were added as powders. Water was added to each diet in a ratio of 3:2 (v/w), and after mixing with a spatula, the diet was lyophilized and then ground in a mortar with a pestle. Individual neonates were placed on 6 mg of diet and held at 28 °C, 16L:8D, ~75% RH over saturated NaCl. After 12 days, each larva was weighed. Growth was calculated from mean initial weight (IW) on day 0 (0.03 mg) and mean final weight (FW) on day 12, according to the formula: (FW-IW/IW). After log transformation of the weights, Ryan's *Q* test was used to determine significant differences between dietary treatments (SAS Institute). The data for percent reduction in growth were not transformed before analysis. The Fisher exact test was used to determine significant differences in mortality.

3. Results

The pH for optimal protein hydrolysis by *T. castaneum* larval gut proteinases was determined for use in the evaluation of inhibitors in vitro. Hydrolysis of a general proteinase substrate, casein, by gut extracts from *T. castaneum* larvae was optimal at pH 4.2 in the presence of a reducing reagent, indicative of cysteine proteinase activity (Fig. 1a). The hydrolysis of more specific substrates, BApNA and SAAPFpNA, provided evidence for both acidic and alkaline proteinases in gut extracts (Fig. 1b,c). BApNA was hydrolyzed maximally at both pH 4.2 and 8.5–9.2, whereas maximal hydrolysis of SAAPFpNA occurred in the alkaline range (pH 9.2–10.6). In contrast, the pH of larval gut extracts was 6.5 ± 0.1 ($n = 3$, ± S.D.).

The effect of selected inhibitors on the activity of *T. castaneum* larval gut proteinases was evaluated in vitro with the general substrate casein in pH 4.2 buffer. Inhibitors were tested over a range of concentrations and compared to the activity measured in the absence of inhibitors (Fig. 2). Under these conditions, the most effective inhibitors were leupeptin, a peptide that inhibits both serine and cysteine proteinases, and E-64, a low molecular weight epoxy derivative with cysteine proteinase selectivity, with IC_{50} values of 0.05 and 0.11 μM, respectively (Table 1). Three proteinaceous cysteine proteinase inhibitors, equistatin, JCPI, and PCPI, caused inhibition similar to that of TLCK and antipain, with IC_{50} values ranging from 0.2–0.8 μM. Chymostatin, TPCK, and STI only weakly inhibited *T. castaneum* gut proteolytic activity, with IC_{50} values of 3.4, 24, and 58 μM,

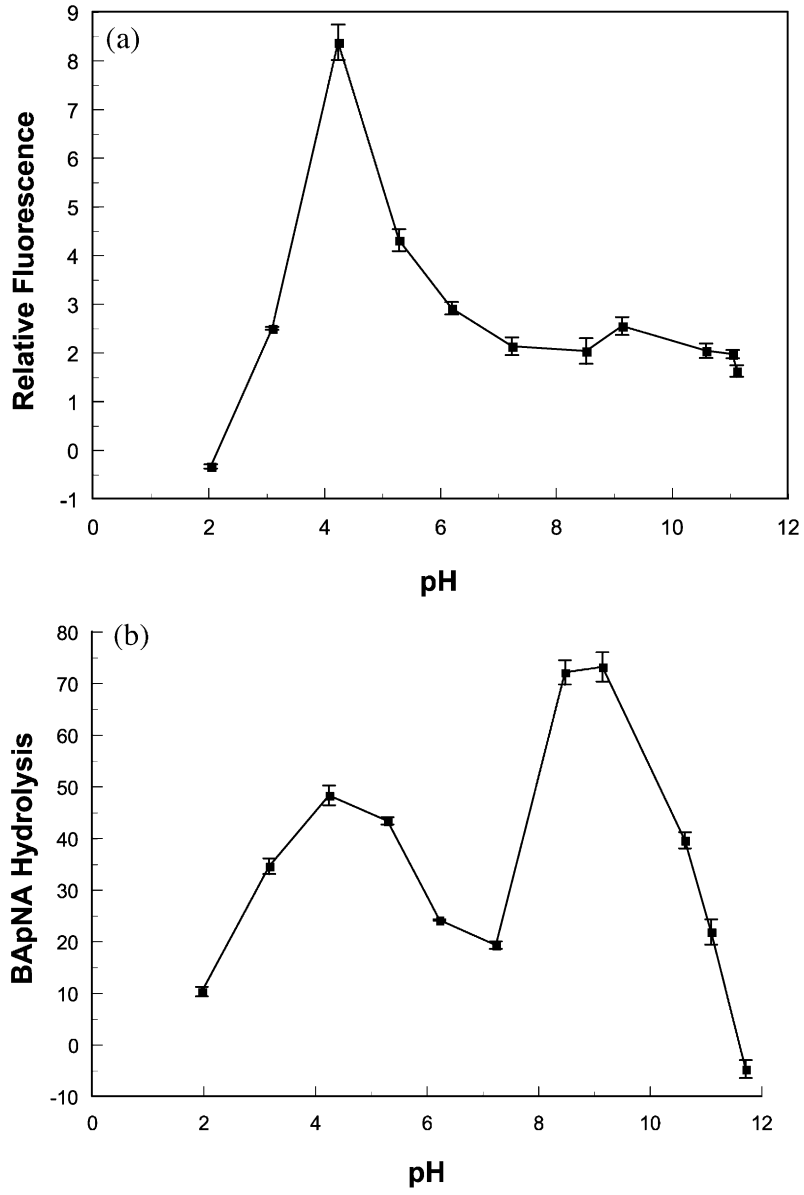

Fig. 1. Effect of pH on the hydrolysis of (a) casein, (b) BApNA, and (c) SAAPFpNA by *Tribolium castaneum* larval gut proteinases. Data represent the mean ± S.D. ($n = 3$).

respectively. Pepstatin at 0.1 mM reduced the caseinolytic activity only approximately 25% (data not shown). Compounds exhibiting little or no inhibition included EDTA, bestatin, and aprotinin (data not shown).

To compare the efficacy of proteinaceous and E-64 inhibitors with *T. castaneum* proteinases in vivo, inhibitors were incorporated into a wheat-based diet of *T. castaneum* larvae. Newly hatched larvae weighed an average of 0.03 mg and increased in mass to 0.7 mg after feeding on the control diet for 12 days (Table 2). There was no significant mortality in any of the treatments. Similar to a previous report (Oppert et al., 1993), 0.1% E-64 was an effective growth inhibitor, and larval growth was 56% less than the control. A combination of 0.1% E-64 with the serine proteinase inhibitor STI at 1% resulted in substantially lower larval weights, with a 91% growth reduction relative to the control.

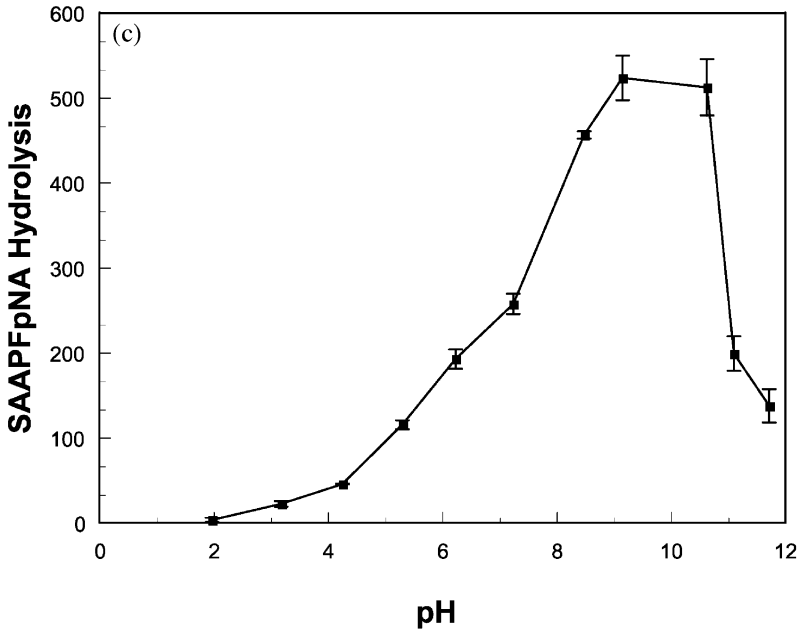

Fig. 1 (Continued).

Two plant cysteine proteinase inhibitors, PCPI and JCPI, and one animal cysteine proteinase inhibitor, equistatin, at 1% were effective in suppressing the weight gain of *T. castaneum* larvae, resulting in 54, 49 and 45% reductions, respectively. On a mole basis, 1% PCPI, JCPI and equistatin in the diet amounted to 2.5, 4.2 and 1.7 nmoles, respectively, which was approximately 1000-fold less than the amount of E-64, 1.5 μmol. The relative potency of the inhibitor (as expressed as the reduction in growth divided by amount of inhibitor) was less for E-64 (0.04) than for JCPI, PCPI, or equistatin (12, 22 and 27, respectively).

In two cases, combinations of inhibitors of cysteine and serine proteinases resulted in significant weight reductions (Table 2). A combination of E-64 and STI resulted in a 91% reduction in growth, and a combination of PCPI and STI caused an 80% reduction in growth. The mean weight of larvae feeding on equistatin was higher than that of larvae fed equistatin combined with STI, although these means were not statistically different. In contrast, the combination of JCPI with STI appeared to be antagonistic, resulting in less inhibition than the treatment with JCPI alone.

4. Discussion

In the present study, we confirmed that the combination of cysteine and serine proteinase inhibitors in the diet of *T. castaneum* larvae exerted a synergistic reduction in growth. The study was extended to other proteinaceous inhibitors to identify genes to incorporate into seed genomes for enhanced protection to storage pests.

In a physiological examination of the *T. castaneum* larval gut, the pH of extracts was 6.5, close to the value of 6.7 reported by Birk and Applebaum (1960). Results from pH-activity profiles using three different substrates revealed the presence of proteinases with acidic (pH 4–5) and alkaline (pH 8.5–11) optima in gut extracts of *T. castaneum*. One interpretation of these data, consistent with inhibitor data, is that the acidic pH optimum corresponded to cysteine proteinase activity, whereas the alkaline optima corresponded to serine proteinase activity. Overall, the predominant caseinolytic activities in *T. castaneum* gut extracts were by proteinases with acidic pH optima. Substrates that were used to further evaluate the alkaline pH optima were preferentially hydrolyzed by trypsin-like (BApNA) or chymotrypsin-

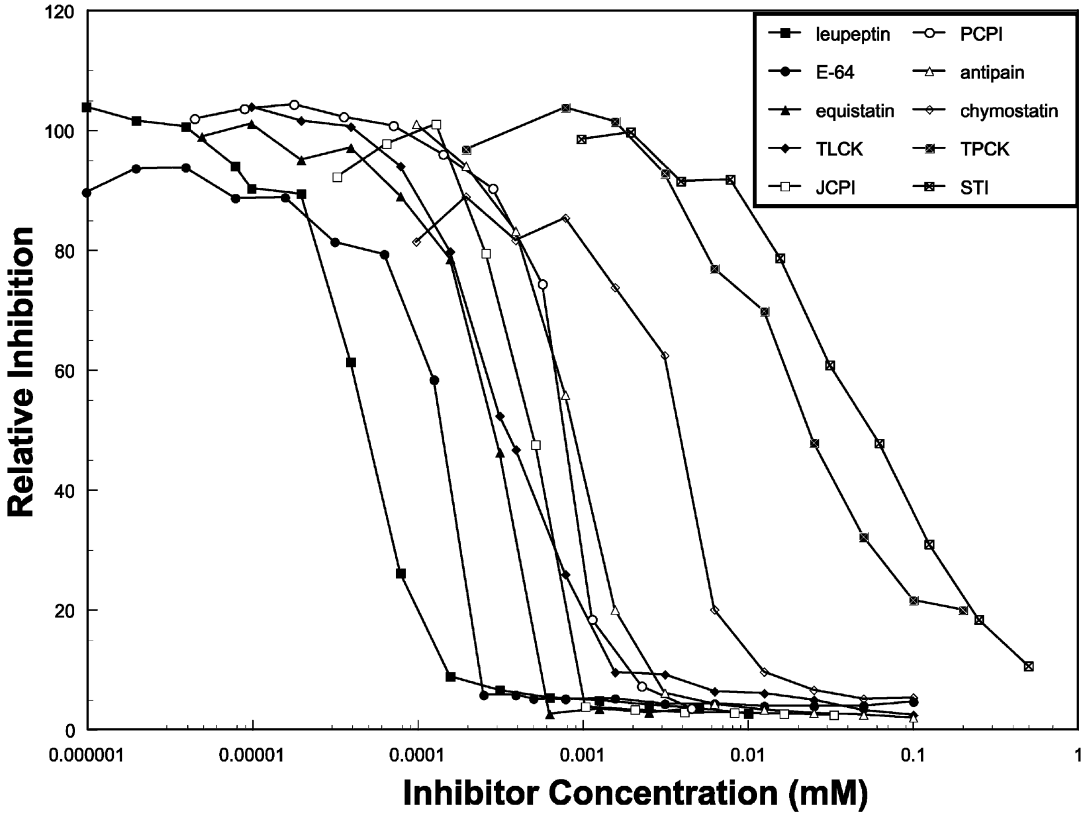

Fig. 2. Relative inhibition (as a percent of the control activity) of casein hydrolysis by *Tribolium castaneum* larval gut proteinases with selected proteinase inhibitors.

Table 2
The effect of proteinase inhibitors in the diet on larval weight of the red flour beetle, *Tribolium castaneum*

Treatment	Larval weight on day 12 (mg)[1]		Percent reduction in growth[2]	Relative potency[3]
	Bioassay I	Bioassay II		
Control	0.73±0.08 (13) a	0.72±0.07 (20) a	–	–
1% STI	0.66±0.05 (14) a	0.63±0.06 (15) a	12±2 a	4.3
0.1% E-64	0.38±0.02 (10) b	0.29±0.02 (20) c	56±6 b	0.04
0.1% E-64 and 1% STI	0.12±0.01 (14) c	0.07±0.01 (10) e	91±4 c	–
1% equistatin	0.47±0.03 (11) ab	0.36±0.06 (8) bc	45±7 b	27
1% equistatin and 1% STI	0.34±0.03 (12) b	–	56	–
1% PCPI	–	0.35±0.04 (12) bc	54	22
1% PCPI and 1% STI	–	0.17±0.04 (11) d	80	–
1% JCPI	–	0.38±0.05 (15) bc	49	12
1% JCPI and 1% STI	–	0.54±0.05 (17) ab	26	–

[1] Mean values±S.E. (number of individuals in parentheses). Data with the same letter within a column were not significantly different according to Ryan's Q test ($P<0.05$).

[2] Percent reduction in growth was calculated from minus the growth of the inhibitor treated larvae divided by that of the control larvae on day 12, x 100.

[3] Relative potency is the percent reduction in growth divided by the nmoles of inhibitor tested, provided for all single inhibitor tests.

like (SAAPFpNA) proteinases. *T. castaneum* proteinases with alkaline pH optima hydrolyzed both BApNA and SAAPFpNA. The magnitude of hydrolysis of SAAPFpNA was much greater than that of BApNA and may indicate that chymotrypsin-like proteinases are the major serine proteinases in *T. castaneum*, as was previously reported in another coleopteran, *L. decemlineata* (Novillo et al., 1997).

Proteinases with an acidic pH optimum also hydrolyzed the substrate BApNA, and it is possible that this hydrolysis was by trypsin-like proteinases. However, acidic cysteine proteinases, such as cathepsin-L, can hydrolyze BApNA, albeit at slower rates than trypsin. Collectively, the data support the hypothesis that cysteine proteinases predominate in the gut digestive proteinase profile of *T. castaneum* larvae, and that serine proteinases play a lesser role in protein digestion.

Previously, we demonstrated that combinations of cysteine and serine proteinase inhibitors were more effective than either inhibitor class singly in reducing the growth and survival of *T. castaneum* larvae (Oppert et al., 1993). However, the cysteine proteinase inhibitor in that study was a small naturally occurring epoxide derivative, E-64, that is produced in multiple steps by a fungus, *Aspergillus japonicus* (Hanada et al., 1978). In the present study, we confirmed that the combination of E-64 with STI in the diet worked synergistically to reduce larval growth. We extended the study to other proteinaceous inhibitors to provide a basis for genetic manipulation of seeds for enhanced protection to storage pests.

Equistatin, a proteinaceous inhibitor from a sea anemone (*A. equina*), is classified as a thyropin, a group of proteins that inhibit both cysteine and cation-dependent proteinases (Lenarcic and Turk, 1999). Equistatin contains domains that inhibit papain-like cysteine proteinases or cathepsin D-like aspartic acid proteinases. Equistatin, as well as other thyroglobulin type-1 domain inhibitors, inhibited the cysteine proteinase activity of *L. decemlineata* (Gruden et al., 1998). In this study, equistatin was one of the most potent proteinaceous inhibitors of the caseinolytic activity in *T. castaneum* gut extracts in vitro. Also, addition of equistatin to *T. castaneum* diets resulted in significantly suppressed larval growth.

JCPI was another proteinaceous cysteine proteinase inhibitor that reduced the larval weight of *T castaneum*. However, the combination of STI with JCPI did not diminished larval growth when compared to effects of JCPI alone. Experiments are in progress to more closely examine the changes in proteinase expression/activity in response to combinations of STI with E-64, PCPI, equistatin, or JCPI.

PCPI was the most effective inhibitor of *T. castaneum* proteinases in this study. The in vitro potency of PCPI with *T. castaneum* proteinases was in the micromolar range, just slightly lower than that of E-64, equistatin, and JCPI. A combination of PCPI with STI was the most effective proteinaceous inhibitor combination in vivo in suppressing larval growth, achieving inhibition levels similar to the E-64/STI combination. The inhibition of growth by PCPI was similar to that of E-64. In vivo potency of PCPI (expressed as the reduction in growth divided by amount of inhibitor) was three orders of magnitude higher than that of E-64. However, each test included only one concentration of each inhibitor. A range of inhibitor concentrations in bioassays is necessary to provide more complete information about inhibitor in vivo potency. These results suggest that PCPI or equistatin combined with a serine proteinase inhibitor, such as STI, is a promising combination for transgenic seed technology to enhance seed resistance to storage pests such as *T. castaneum*.

There have been several other reports of the in vivo and in vitro effects of proteinase inhibitors on the red flour beetle. Birk and Applebaum (1960) reported that partially purified proteinase inhibitors from soybeans inhibited the growth of *T. castaneum* larvae. Further purification, however, resulted in the separation of trypsin and chymotrypsin inhibitors from those that lack serine proteinase activity (Birk et al., 1963). It has been speculated that the inhibition of *T. castaneum* larval growth by the partially purified soybean inhibitors was by a soybean cysteine proteinase inhibitor (Hines et al., 1991). A cysteine proteinase inhibitor from rice, oryzacystatin, fed to flour beetle larvae at a dietary concentration of 10%, suppressed growth by approximately 35% (Chen et al., 1992). In this study, proteinaceous inhibitors fed at only 1% in the diet exhibited similar effects on growth. Oryzacystatin inhibited *T. castaneum* caseinolytic activity with an IC_{50} value of 2 μM at pH 6 (Liang et al., 1991; Chen et al., 1992). This concentration is similar to IC_{50} values (1

μM) reported in this study for related inhibitors from Job's tears and potato.

Our original hypothesis was that the insecticidal synergy observed with combinations of cysteine and serine proteinase inhibitors towards *T. castaneum* proteinases was due to an inhibition of two classes of proteinases in this coleopteran (Oppert et al., 1993). However, some insects have an adaptive phenotypic plasticity that provides compensation for ingested inhibitors by increasing the production of insensitive proteinases (see Oppert, 2000 for a review; Agrawal, 2001). Therefore, another explanation for the synergy of cysteine and serine proteinase inhibitors in bioassays with *T. castaneum* larvae is that the combination of inhibitors diminishes the adaptive response. Our results suggest that a combination of PCPI or equistatin with a serine proteinase inhibitor, such as STI, may enhance seed resistance to storage pests such as *T. castaneum*.

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References

Agrawal, A.A., 2001. Phenotypic plasticity in the interaction and evolution of species. Science 294, 321–326.

Baker, J.E., 1982. Application of capillary thin layer isoelectric focusing in polyacrylamide gel to the study of alkaline proteinases in stored-product insects. Comp. Biochem. Physiol. Part B 71, 501–506.

Birk, Y., Applebaum, S.W., 1960. Effect of soybean trypsin inhibitors on the development and midgut proteolytic activity of *Tribolium castaneum* larvae. Enzymologia 22, 318–326.

Birk, Y., Gertler, A., Khalef, S., 1963. Separation of a Tribolium-protease inhibitor from soybeans on a calcium phosphate column. Biochim. Biophys. Acta 67, 324–326.

Blanco-Labra, A., Martinez-Gallardo, N.A., Sandoval-Cardoso, L., Delano-Frier, J., 1996. Purification and characterization of a digestive cathepsin D proteinase isolated from *Tribolium castaneum* larvae (Herbst). Insect Biochem. Biol. 26, 95–100.

Brunelle, F., Nguyen-Quoc, B., Cloutier, C., Michaud, D., 1999. Protein hydrolysis by Colorado potato beetle, *Leptinotarsa decemlineata*, digestive proteases: the catalytic role of cathepsin D. Arch. Insect Biochem. Physiol. 42, 88–98.

Brzin, J., Popovic, T., Drobnic-Kosorok, M., Kotnik, M., Turk, V., 1988. Inhibitors of cysteine proteinases from potato. Biol. Chem. Hoppe-Seyler 369, 233–238.

Carlini, C.R., Oliveira, A.E., Azambuja, P., Xavier-Filho, J., Wells, M.A., 1997. Biological effects of canatoxin in different insect models: evidence for a proteolytic activation of the toxin by insect cathepsin-like enzymes. J. Econ. Entomol. 90, 340–348.

Chen, M.-S., Johnson, B., Wen, L., Muthukrishnan, S., Kramer, K.J., Morgan, T.D., Reeck, G.R., 1992. Rice cystatin: bacterial expression, purification, cysteine proteinase inhibiting activity and insect growth inhibiting activity of a truncated form of rice cystatin. Protein Express. Purification 3, 41–49.

Cuperus, G, Krischik, V., 1995. Why stored product integrated pest management is needed. Stored Product Management. Oklahoma State Univ. Coop. Ext. Serv. Circ. E-912, pp. 199.

Edmonds, H.S., Gatehouse, L.N., Hilder, V.A., Gatehouse, J.A., 1996. The inhibitory effects of the cysteine protease inhibitor, oryzacystatin, on digestive proteases and on larval survival and development of the southern corn rootworm (*Diabrotica undecimpunctata* howardi). Entomologia Exp. Appl. 78, 83–94.

Fabrick, J., Behnke, C., Czapla, T., Bala, K., Rao, A.G., Kramer, K.J., Reeck, G.R., 2002. Effects of a potato cysteine proteinase inhibitor on midgut proteolytic enzyme activity and growth of the southern corn rootworm, *Diabrotica undecimpunctata* howardi (Coleoptera: Chrysomelidae). Insect Biochem. Mol. Biol. 32, 405–415.

Frugoni, J.A.C., 1957. Tampone universale di Britton e Robinson a forza ionica costante. Gazz. Chem. Ital. 87, 403–407.

Gillikin, J., Bevilacqua, S., Graham, J.S., 1992. Partial characterization of digestive tract proteinases from western corn rootworm larvae, *Diabrotica virgifera*. Arch. Insect Biochem. Physiol. 19, 285–298.

Girard, C., Le Métayer, M., Bonadé-Bottino, M., Pham-Delegue, M.-H., Jouanin, L., 1998. High level of resistance to proteinase inhibitors may be conferred by proteolytic cleavage in beetle larvae. Insect Biochem. Mol. Biol. 28, 229–237.

Gruden, K., Strukelj, B., Popovic, T., Lenarcic, B., Bevec, T., Brzin, J., Kregar, I., Herzog-Velikonja, J., Steiekema, W.J., Bosch, D., Jongsma, M.A., 1998. The cysteine protease activity of the Colorado potato beetle (*Leptinotarsa decemlineata* Say) guts, which is insensitive to potato protease inhibitors, is inhibited by thyroglobulin type-1 domain inhibitors. Insect Biochem. Mol. Biol. 28, 549–560.

Hanada, K., Tamai, M., Yamagishi, M., Ohmura, S., Sawada, J., Tanaka, I., 1978. Isolation and characterization of E-64, a new thiol protease inhibitor. Agric. Biol. Chem. 42, 523–528.

Harein, P, Meronuck, R., 1995. Stored grain losses due to insects and molds and the importance of proper grain management. Stored Product Management. Oklahoma State Univ. Coop. Ext. Serv. Circ. E-912, pp. 29–31.

Hilder, V.A., Boulter, D., 1999. Genetic engineering of crop plants for insect resistance—a critical review. Crop Prot. 18, 177–191.

Hines, M.E., Osuala, C.I., Nielsen, S.S., 1991. Isolation and partial characterization of a soybean cystatin cysteine proteinase inhibitor of coleopteran digestive proteolytic activity. J. Agric. Food Chem. 39, 1515–1520.

Koiwa, H., Shade, R.E., Zhu-Salzman, K., D'Urzo, M.P., Murdock, L.L., Bressan, R.A., Hasegawa, P.M., 2000. A plant defensive cystatin (soyacystatin) targets cathepsin L-like digestive cysteine proteinases (DvCALs) in the larval midgut of western corn rootworm (*Diabrotica virgifera virgifera*). FEBS Lett. 471, 67–70.

Kolterman D, Thiessen EJ, Wells E. 2000. Kansas on-farm postharvest chemical usage. Kansas Agricultural Statistics Fact Finders for Agriculture, Kansas Department of Agriculture, Apr; 1–4.

Krizaj, I., Drobnic-Kosorok, M., Brzin, J., Jerala, R., Turk, V., 1993. The primary structure of inhibitor of cysteine proteinases from potato. FEBS Lett. 333, 15–20.

Lenarcic, B., Ritonja, A., Strukelj, B., Turk, B., Turk, V., 1997. Equistatin, a new inhibitor of cysteine proteinases from *Actinia equina*, is structurally related to thyroglobulin type-1 domain. J. Biol. Chem. 272, 13899–13903.

Lenarcic, B., Turk, V., 1999. Thyroglobulin type-1 domains in equistatin inhibit both papain-like and cysteine proteinases and cathepsin D. J. Biol. Chem. 274, 563–566.

Liang, C., Brookhart, G., Feng, G.H., Reeck, G.R., Kramer, K.J., 1991. Inhibition of digestive proteinases of stored grain Coleoptera by oryzacystatin, a cysteine proteinase inhibitor from rice seed. FEBS Lett. 2, 139–142.

Michaud, D., Nguyen-Quoc, B., Vrain, T., Fong, D., Yelle, S., 1996. Response of digestive cysteine proteinases from the Colorado potato beetle (*Leptinotarsa decemlineata*) and the black vine weevil (*Otiorynchus sulcatus*) to a recombinant form of human stefin A. Arch. Insect Biochem. Physiol. 31, 451–464.

Novillo, C., CastaZera, P., Ortego, F., 1997. Characterization and distribution of chymotrypsin-like and other digestive proteases in Colorado potato beetle larvae. Arch. Insect Biochem. Physiol. 36, 181–201.

Oppert, B., 2000. Transgenic plants expressing enzyme inhibitors and the prospects for biopesticide development. In: Koul, O., Dhaliwal, G.S. (Eds.), Phytochemical Biopesticides. Harwood Academic, The Netherlands, pp. 83–95.

Oppert, B., Hartzer, K., Smith, C.M., 2000. Characterization of the digestive proteinases of *Hypera postica* (Gyllenhal) (Coleoptera: Curculionidae). Trans. Kansas Acad. Sci. 103, 99–110.

Oppert, B., Kramer, K.J., McGaughey, W.H., 1997. Rapid microplate assay of proteinase mixtures. BioTechniques 23, 70–72.

Oppert, B., Morgan, T.D., Culbertson, C., Kramer, K.J., 1993. Dietary mixtures of cysteine and serine proteinase inhibitors exhibit synergistic toxicity toward the red flour beetle, *Tribolium castaneum*. Comp. Biochem. Physiol. Part C 105, 379–385.

Pernas, M., Sanchez-Monge, R., Gomez, L., Salcedo, G., 1998. A chestnut seed cystatin differentially effective against cysteine proteinases from closely related pests. Plant Mol. Biol. 38, 1235–1242.

Reeck, G.R., Kramer, K.J., Baker, J.E., Kanost, M., Fabrick, J., Behnke, C.A., 1997. Proteinase inhibitors and resistance of transgenic plants to insects. In: Carrozi, N.B., Koziel, M.G. (Eds.), Advances in Insect Control: The Role of Transgenic Plants. Taylor & Francis Ltd, London, pp. 157–183.

Reeck, G., Oppert, B., Denton, M., Kanost, M., Baker, J., Kramer, K., 1999. Insect proteinases. In: Turk, V. (Ed.), Proteases, New Perspectives. Birkhauser, Basel, Switzerland, pp. 125–148.

Ritonja, A., Turk, B., Turk, V., 1997. Equistatin, a new inhibitor of cysteine proteinases from *Actinia equina*, is structurally related to thyroglobulin type-1 domain. J. Biol. Chem. 272, 13899–13903.

Yoza, K., Nakamura, S., Haraguchi, K., Ohtsubo, K., 2001. Molecular cloning of a cysteine proteinase inhibitor (cystatin) of Job's tears (*Coix lachryma-Jobi* L.). In: Pavlath, A.E., Cherry, J.P. (Eds.), Proc. XXIXth Annual Meeting US–Japan Coop. Prog. Nat. Resources Protein Panel. HH6, pp. H1–H.